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Rapid Secretion of Interleukin-1β by Microvesicle Shedding

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Summary

The proinflammatory cytokine interleukin-1β (IL-1β) is a secreted protein that lacks a signal peptide and does not follow currently known pathways of secretion. Its efficient release from activated immune cells requires a secondary stimulus such as extracellular ATP acting on $P2X_7$ receptors. We show that human THP-1 monocytes shed microvesicles from their plasma membrane within 2–5 s of activation of $P2X_7$ receptors. Two minutes after such stimulation, the released microvesicles contained bioactive IL-1β, which only later appeared in the vesicle-free supernatant. We conclude that microvesicle shedding is a major secretory pathway for rapid IL-1β release from activated monocytes and may represent a more general mechanism for secretion of similar leaderless secretory proteins.

Introduction

The cytokine IL-1β is the key initiator of the acute inflammatory response (Dinarello, 1996), but the mechanism of its release from cells is enigmatic. This protein lacks a secretory signal sequence and does not follow the classical endoplasmic reticulum-to-Golgi pathway of secretion (Rubartelli and Sitia, 1997), nor is there any indication that it is stored in or released from exocytotic granules, although a small fraction of this cytokine has been found in secreted endolysosomes (Andrei et al., 1999). It is synthesized in immune cells of monocytic origin as a biologically inactive 31 kDa precursor molecule that is cleaved to its active 17 kDa form by IL-1β-converting enzyme (ICE or caspase-1) (Dinarello, 1996; Zeuner et al., 1999). Cleavage of proIL-1β is tightly coupled to its release because processed IL-1β can only be detected extracellularly (Dinarello, 1996; Hogquist et al., 1991). An initial inflammatory stimulus such as bacterial endotoxin initiates synthesis of both pro-caspase-1 and pro-IL-1β (i.e., the caspase cascade), but in the absence of a secondary stimulus, processing and release of IL-1β are inefficient, with most of the newly synthesized products remaining intracellular and unprocessed or being degraded (Hogquist et al., 1991; Perregaux and Gabel, 1994; Ferrari et al., 1997; Sanz and Di Virglio, 2000, Di Virgilio et al., 1998). The only known physiological stimuli for the processing and immediate release of bioactive IL-1β are cytotoxic T cells and extracellular ATP acting on purinergic $P2X_7$ receptors (Hogquist et al., 1991; Perregaux and Gabel, 1994; Ferrari et al., 1997; Sanz and DiVirgilio, 2000, Gardella et al., 2000).

$P2X_7$ receptors belong to a family of ion channels gated by extracellular ATP (Mackenzie et al., 1999). $P2X_{1-6}$ receptors are widely distributed in both neuronal and nonneuronal cells, while $P2X_7$ receptors are not found in neurones but are highly expressed in immune and epithelial cells (Ralevic and Burnstock, 1998). A unique feature of several of these ion channels is their ability to change their ion selectivity during receptor activation (Surprenant et al., 1996; Virginio et al., 1999a; Khakh et al., 1999). The channel that opens initially (i.e., within several milliseconds) is permeable only to small cations, including calcium; however, if the ATP application is prolonged for 10–30 s, some P2X receptors ($P2X_2$, $P2X_4$, $P2X_{2/3}$, and $P2X_7$) become permeable to larger cations up to approximately 600–900 Da. Although the molecular and cellular mechanisms underlying this pore dilatation are unknown, pore dilatation per se cannot fully account for the even more dramatic morphological changes that occur during and subsequent to activation of $P2X_7$ receptors but not the other P2X receptors. That is, activation of $P2X_7$ receptors results in extensive cell membrane blebbing within seconds to minutes, and sustained activation of the receptor for $>$30 min results in eventual cell death 6–24 hr later as assayed by methods such as trypan blue uptake and release of lactate dehydrogenase (LDH) or chromium (Hogquist et al., 1991; Ferrari et al., 1997; Sanz and DiVirgilio, 2000, Virginio et al., 1999b). Prior to the recent biophysical demonstrations that pore dilatation is the same at the $P2X_2$ and $P2X_7$ receptors (Virginio et al., 1999a, 1999b; Khakh et al., 1999; Khakh and Lester, 1999), it had been assumed that the cell death occurring after sustained $P2X_7$ receptor activation was a direct result of pore dilatation resulting in eventual osmotic shock and cell lysis (Hogquist et al., 1991; DiVirgilio et al., 1998). Activation of $P2X_2$ receptors expressed in HEK cells or present in sensory or autonomic neurones, however, never results in membrane blebbing and/or subsequent cell death, even during application of ATP for several hours (Virginio et al., 1999b; Khakh et al., 1999; Khakh and Lester, 1999). These results call into question previous suggestions that IL-1β release evoked by activation of $P2X_7$ receptors may be due to apoptotic and/or necrotic cell death via pore dilatation sequelae.

Many studies have concluded that it is the $P2X_7$ receptor that is responsible for the ATP-mediated IL-1β release from activated monocytes, macrophage, and microglia (DiVirgilio et al., 1998); this has now been confirmed by results from transgenic mice lacking the $P2X_7$ receptor where monocytes primed with the bacterial endotoxin lipopolysaccharide no longer release IL-1β in response to application of ATP nor do these cells die during prolonged ATP application (Solle et al., 2001). This apparent correlation between $P2X_7$ receptor-mediated IL-1β release and cell death appears to support the concept that ATP-mediated aponecrosis may indeed be responsible for release of IL-1β whether or not the underlying mecha-

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nism involves pore dilatation. However, previous studies have measured IL-1β release and cell death criteria only after prolonged (≥30 min) $P2X_7$ receptor activation. Because membrane blebbing is a commonly accepted extranuclear marker of a cell undergoing apoptosis and/or necrosis (Wyllie et al., 1980; Majno and Joris, 1995) and because significant membrane blebbing occurs in the first few minutes of $P2X_7$ receptor activation, the original aim of the present study was to determine whether this apparently rapid aponecrosis might be associated with a similarly rapid release of IL-1β from activated monocytes.

Toward this aim, we examined HEK cells heterologously expressing the $P2X_7$ receptor and human monocyte THP-1 cells; these are known to initiate the caspase cascade and release bioactive IL-1β in response to $P2X_7$ receptor activation after lipopolysaccharide priming (Humphreys and Dubyak, 1998; Grahames et al., 1999). To our surprise, we found that bioactive IL-1β was released within the first 2 min of $P2X_7$ receptor activation with no concomitant cell death. This cytokine was contained within microvesicles (<0.5 μm diameter) that were shed from the plasma membrane within seconds of $P2X_7$ receptor activation. Rapid plasma membrane shedding of microvesicles containing IL-1β has not been demonstrated previously and represents a new secretory pathway that may possibly underlie release of similar leaderless secretory proteins.

Results

No Cell Death Occurs During Initial 30 Min of Sustained $P2X_7$ Receptor Activation

Because of the widely held view that IL-1β is liberated as a consequence of cell death, we used several different approaches to assess this within the first 30 min after applying extracellular ATP or the related agonist benzoyl-(4-benzoyl)-ATP (BzATP).

Biophysical Measurements

We made whole-cell recordings from HEK cells (n = 51) expressing the $P2X_7$ receptor (Surprenant et al., 1996) and from human THP-1 monocytes which had (n = 14) or had not (n = 21) been exposed to lipopolysaccharide (LPS). The kinetics of $P2X_7$-evoked currents recorded from HEK cells during very brief (1–30 s) applications of ATP or BzATP have been described in detail previously (Surprenant et al., 1996; Virginio et al., 1999b; Hibell et al., 2000). In THP-1 monocytes, the peak amplitude of the current evoked by BzATP (300 μM) varied greatly in cells that had not been exposed to LPS (40–2100 pA, mean = 500 ± 131 pA) but not in LPS-primed cells (1300–2360 pA, mean 1800 ± 88 pA), with no significant change in surface area as measured by membrane capacitance (37 ± 3 pF versus 35 ± 4 pF) or obvious differences in overall cell morphology. The significant increase in BzATP-evoked currents in LPS-primed monocytes is most likely due to increased $P2X_7$ receptor expression in view of previous work that showed significant increases in $P2X_7$ mRNA levels in these cells after LPS treatment (Humphreys and Dubyak, 1998). Prolonged application of BzATP for 1–10 min elicited a sustained inward cationic current which was readily reversible in normal extracellular solution (Figure 1B). However, within 30–90 s of applying BzATP to HEK cells, the plasma membrane exhibited extensive bleb formation (Figures 1A and 1C; see Supplemental Movie S1 at http://www.immunity.com/cgi/content/full/15/5/825/DC1). THP-1 cells showed a much more variable time to onset of initial membrane blebbing, from 90–480 s in LPS-primed cells and 90–500 s in nonprimed cells. The appearance of blebs was not associated with any loss of membrane seal during patch clamp recordings for up to 2 hr subsequently (n = 5 HEK cells and 8 THP-1 monocytes) and was not due to osmotic shock because it was not prevented by hypertonic solutions (400–600 mOsm, n = 4).

Biochemical Measurements

During continuous application of BzATP, the dye BCECF (520 Da) was retained in the cell, trypan blue was excluded from HEK cells for up to 40 min and more than 4 hr from THP-1 cells, and no LDH was detected in medium collected from HEK or THP-1 cells exposed to BzATP for 5, 10, or 30 min (n = 3). A previous study has also shown that LDH is not released from LPS-treated and nontreated THP-1 cells during 4 hr of continuous BzATP application (Grahames et al., 1999).

Annexin-V Binding Assay for Apoptosis

Annexin-V is a high-affinity phosphatidylserine (PS) binding protein which is widely used as a marker of cells destined for, or in the execution phase of, cell death by apoptosis because translocation of PS from the inner leaflet of the plasma membrane to the outer (PS flip) is a general feature of such apoptotic cells (Majno and Joris, 1995). We applied fluorescein-conjugated annexin-V to HEK cells prior to and at 2, 10, and 30 min of $P2X_7$ receptor activation with BzATP (100 μM); prior to agonist application <2% of cells were annexin-positive while >95% of cells were annexin-positive at all three of the time points examined (Figure 2). However, there was no subsequent loss of cells over 24 hr after the 2 min application of BzATP and PS flip completely *reversed* within 3 hr (Figure 2A). A similar reversal of the PS translocation without subsequent cell death was observed when $P2X_7$ receptor activation was limited to 10 min (data not shown). In contrast, prolonged BzATP application (30 min) resulted in *irreversible* PS flip and virtual loss of all cells within 6 hr (Figure 2B).

In brief, these experiments show that application of BzATP for up to 10 min results in an inward cation current, extensive membrane blebbing, and PS flip, but it did not lead to cell death.

Rapid Plasma Membrane Vesicle Shedding Occurs within Seconds of $P2X_7$ Receptor Stimulation

Capacitance Measurements

Membrane capacitance recorded after a 2 min application of BzATP was decreased by 22% ± 2% in HEK cells (range 6%–46%, n = 28) and by 34% ± 6% (range 3%–46%, n = 8) in THP-1 monocytes (Figure 3A). The drop in cell capacitance did not progress significantly after the initial 2–5 min of agonist application (Figure 3B); neither did it recover in 30–45 min subsequent to agonist application (n = 6). The decrease in membrane capacitance was blocked by removal of extracellular calcium (Figure 3A) even though membrane blebbing

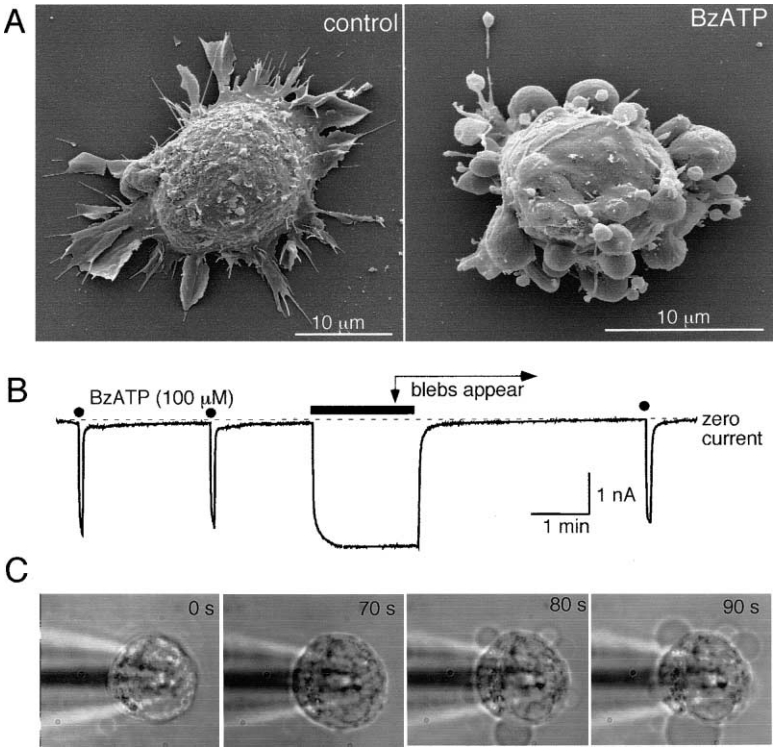

Figure 1. Activation of $P2X_7$ Receptors Causes Membrane Blebbing but Not Cell Lysis

(A) Scanning electron microscopic photos of HEK cells expressing rat $P2X_7$ receptor; control cell shows typical morphology with rough surface and both fine filopodia and sheet-like lamellipodia while cell exposed to BzATP for 2 min shows smooth surface and numerous large (>1 μm) blebs and small (<0.5 μm) microvesicles.

(B) The membrane current recorded from HEK cell during very brief (3 s, at filled circles) and sustained (90 s, at gray bar) application of BzATP; note that the current quickly returns to baseline after prolonged BzATP even though extensive membrane blebbing (C) occurs after about 70 s of receptor activation.

was not. However, it was still observed in the absence of extracellular sodium (capacitance decrease after 5 min BzATP was 31% ± 5% and 39% ± 8% in HEK and THP-1 cells, respectively; n = 4), which prevented the early formation of blebs (Virginio et al., 1999a). No significant change in membrane capacitance occurred in time-control recordings from unstimulated HEK cells nor in $P2X_2$-transfected HEK cells exposed to ATP (100 μM) for up to 10 min (Figure 3A), nor did these cells exhibit ATP-evoked blebbing. These experiments indicate a significant loss in membrane surface area following a 2 min activation of the $P2X_7$ receptor and suggest that this is unrelated to the formation of membrane blebs.

Microscopy

This irreversible loss of plasma membrane and the observation of microvesicles (<0.5 μm diameter) under scanning electron microscopy (Figure 1A) suggested a shedding of microvesicles which would be below the resolution of standard bright-field microscopy (e.g., see Supplemental Movie S1 at http://www.immunity.com/cgi/content/full/15/5/825/DC1). By fluorescence video microscopy and nitrobenzoxadiazole (NBD)-conjugated lipid to label the plasma membrane (Chattopadhyay, 1990), we clearly observed shedding of microvesicles within 4–20 s of applying BzATP (Figure 4). The distinctive pinching off of these microvesicles from the plasma membrane is readily visualized in Supplemental Movies S2 and S3, which are typical examples recorded from THP-1 monocytes (n = 54) and HEK cells (n = 42), respectively. Like the decrease in membrane capacitance, microvesicle shedding also required extracellular calcium (n = 30 HEK and 24 THP1 cells; Figures 4B and 4C). Microvesicle shedding from LPS-primed and nonprimed THP-1 cells was qualitatively similar. Shedding of NBD-labeled vesicles was never observed in nontransfected or $P2X_2$ receptor-transfected HEK cells during application of BzATP (200 μM) or ATP (100 μM; see Supplemental Movie S4).

PS Flip Precedes Vesicle Shedding and PS Is Exposed on Shed Microvesicles

We next examined the kinetics of the reversible PS flip we had observed after brief 2–10 min exposure to BzATP (Figure 2) in order to determine whether it was associated with the rapid microvesicle shedding. Using the fluorescein-conjugated annexin-V and video imaging, we detected exposure of PS on the outer leaflet of the plasma membrane as early as 1–2 s after application of BzATP. The microvesicles that were subsequently shed were labeled with annexin (Figure 5A, see Supplemental Movie S5), thus showing that PS was also exposed on their outer membrane. We were not able to determine whether the PS flip was dependent on extracellular calcium because annexin-V binds specifically to PS only in the presence of calcium (Wiedmer et al., 1990; Andree et al., 1990). FM1-43, which has been used to assess PS translocation in the absence of calcium (Zweifach, 2000) rapidly entered the cell through the large pore induced by BzATP resulting in fluorescence of intracellular organelles and the cell nucleus (data not shown). This result is not unexpected in view of its similarity in size and charge to other fluorescent dyes such as YO-PRO-1 which are commonly used to assess pore formation in cells expressing $P2X_7$ receptors (Mackenzie et al., 1999; Virginio et al., 1999b; Hibell et al., 2000).

A comparison of the relative times to onset of annexin binding, NBD-labeled membrane vesicle shedding, and membrane blebbing shows that annexin binding pre-

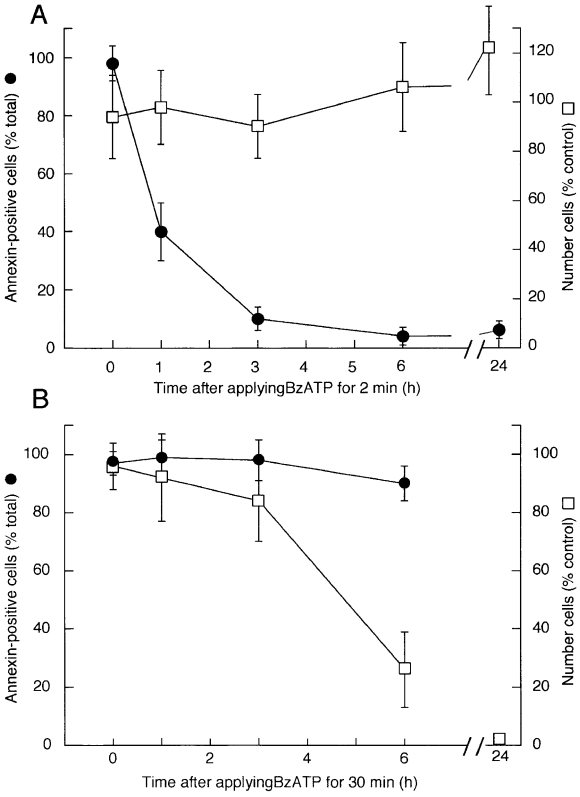

Figure 2. PS Flip Is Reversible and Not Associated with Cell Death after Brief, but Not Prolonged, Receptor Stimulation

HEK cells were exposed to BzATP for (A) 2 min or (B) 30 min; FITC-conjugated annexin (5 μl/100 μl solution) was applied 0, 1, 3, 6, or 24 hr later, and annexin-positive cells (circles; expressed as percent of total) and total number of cells (squares; expressed as percent of time-matched controls) were counted (n = 3 for each time point). No 24 hr time point for annexin binding is shown in (B) because no cells were present.

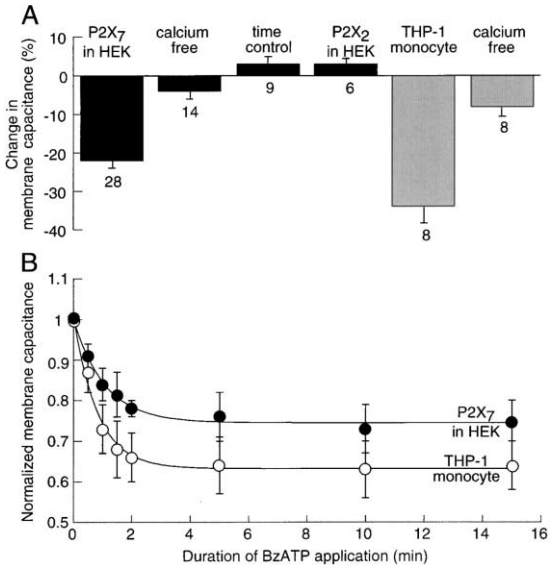

Figure 3. Activation of $P2X_7$ Receptors Causes Loss of Plasma Membrane

(A) The change in membrane capacitance in HEK cells (black) and THP-1 monocytes (gray) after a 2 min exposure to BzATP; the numbers below each bar are numbers of cells.

(B) The decrease in membrane capacitance plateaus with increasing duration of receptor activation. The graph plots membrane capacitance as a function of time of BzATP application in HEK cells (filled circles) or monocytes (open circles); each application of BzATP was followed by a 5–7 min wash. Except for the 10 min time points (see text), n = 4–7 for each point. The lines are best-fit single exponential functions with time constants of 1.1 min (HEK) and 0.7 min (THP1), respectively.

cedes vesicle shedding by 2–10 s while membrane blebbing does not occur until some 40 s (in HEK cells) to 150 s (in monocytes) later (Figure 5B).

Shed Microvesicles Contain IL-1β

We asked whether IL-1β was present in microvesicles shed from activated THP-1 monocytes by isolating microvesicles released into the supernatant with annexin-coated beads and then assaying for the presence of IL-1β in both vesicular and vesicle-free supernatant fractions. IL-1β was detected in the microvesicles at the earliest time point (1–2 min, n = 6/7 experiments) after $P2X_7$ receptor activation and increased in an approximately exponential manner with a time constant of 5.6 ± 1 min (n = 6, Figure 6A). This plateau in the release of microvesicles is similar to our results with decreases in membrane capacitance that also reached a steady value after 2–5 min of continuous receptor activation and suggests the possibility of a readily releasable pool of microvesicles. Secretion experiments showed that, while IL-1β was consistently found in the microvesicle fraction at all times points <10 min, its presence in the microvesicle-free fraction was quite variable for these shorter times (Figure 6A). When data from all individual experiments were normalized to the amount of IL-1β measured at the 30 min time point, IL-1β was significantly greater in the microvesicle-containing than in the microvesicle-free fraction for all times points <30 min (Figure 6B). Significant levels of IL-1β were only detected in the microvesicle fraction after triton treatment to release vesicle contents; no IL-1β (±12% of background, n = 3) was detected in this fraction in the absence of triton, thus demonstrating that the cytokine was contained within the microvesicles. Moreover, there was no significant difference in IL-1β content for microvesicles that were incubated with high concentrations of trypsin (n = 4), showing that the initially released IL-1β was insensitive to protease digestion and further adding to the conclusion that the cytokine was intravesicular.

We considered the possibility that the IL-1β that we measured was due to lysis of a small subset of cells either by the BzATP or LPS stimuli or by triton treatment of cells present in the collected medium. We discounted this because no trypan blue-positive cells were observed immediately after collecting the medium and microscopic inspection of the total medium collected prior to separation into vesicle and vesicle-free fractions showed at most one cell, irrespective of duration or presence of BzATP or LPS. Furthermore, the amount of IL-1β measured in triton-treated supernatant (i.e., total extracellular IL-1β) at the 10 and 30 min time points was 12% and 28%, respectively of the total cellular content (340 pg/10^5 cells versus 2900 pg/10^5 cells at 10 min and

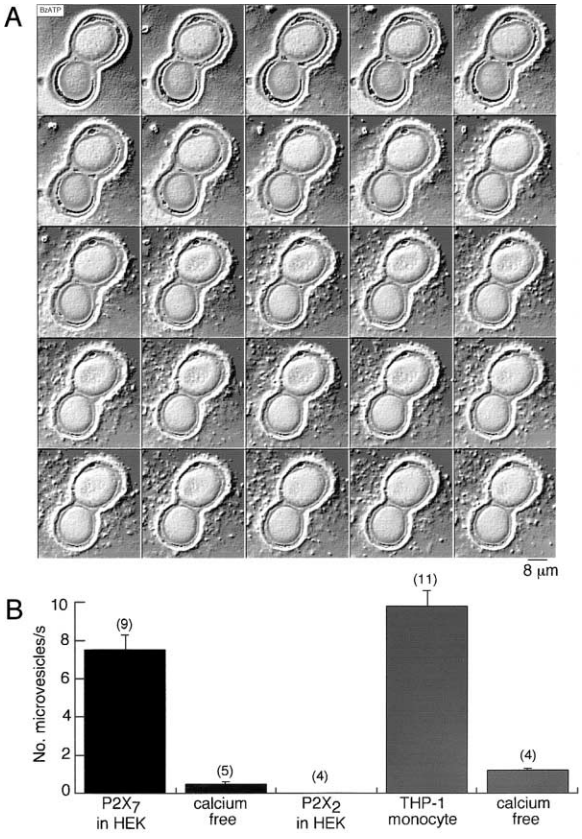

Figure 4. Rapid Microvesicle Shedding Following $P2X_7$ Receptor Activation

(A) Fluorescent images of two THP-1 monocytes labeled with NBD during exposure to BzATP; the frames are shown at 2 s intervals from the time of BzATP application (Frame 1). Images have been shadowed with NIH Image 1.62 to accentuate the small vesicles budding from the plasma membrane which can be seen clearly in Supplemental Movie S2 (THP-1 monocyte) and Supplemental Movie S3 (HEK cell).

(B) The kinetics of microvesicle release following $P2X_7$ receptor stimulation in HEK and THP-1 cells; individual fluorescent particles that were >5 μm distance from the plasma membrane were counted.

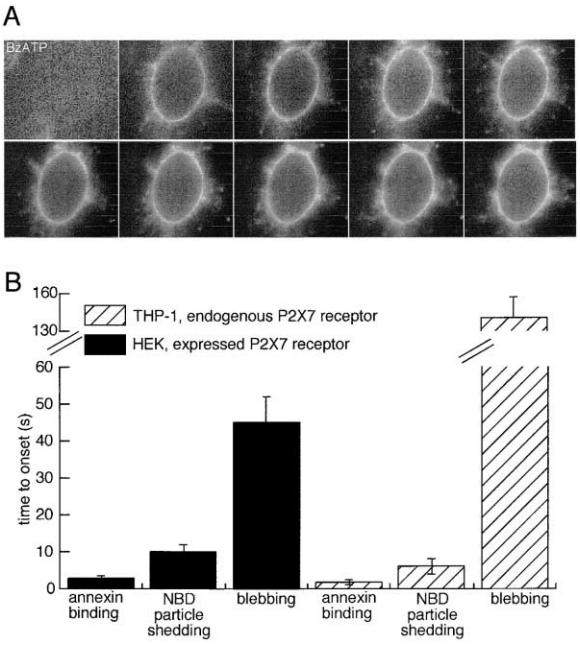

Figure 5. PS Flip Is Rapid and Precedes Microvesicle Shedding

(A) Fluorescent images of HEK cell during BzATP exposure (from Frame 1, images at 5 s intervals are shown) in the presence of FITC-conjugated annexin V. Annexin V (5 μl/100 μl solution) was present for 2 min prior to BzATP without labeling the cell but labeling occurs within 1 s of receptor activation, after which annexin-labeled microvesicle shedding is apparent (see Supplemental Movie S5).

(B) The graph shows a summary of all the experiments as illustrated in Supplemental Movies S1, S3, and S5. The time to onset (from the frame in which BzATP was added) for annexin binding to plasma membrane, NBD-labeled particle shedding, and membrane blebbing in HEK and THP-1 cells are indicated. Data are mean ± SEM of 5–12 cells.

520 pg/10^5 cells versus 1860 pg/10^5 cells at 30 min from separate experiments). These values are similar to those obtained recently by Sanz and Di Virgilio (2000) who found released IL-1β was 25% of cellular IL-1β content after 30 min exposure to BzATP in similarly LPS-primed microglia and conclusively ruled out any possibility that the released IL-1β was due to lysis of a small proportion of cells or release of cytosolic contents. Moreover, no LDH could be detected after triton treatment of microvesicles or the microvesicle-free supernatant collected 5 or 10 min after BzATP stimulation (n = 2), thus demonstrating that nonspecific cytosolic proteins are not contained within these microvesicles. A similar result was obtained in cells transfected with cytoplasmic GFP, in that we observed no loss of fluorescence in GFP-expressing HEK cells (n = 35) or LPS-treated THP-1 cells (n = 12) during exposure to BzATP for 2–10 min. In THP-1 cells which had not been previously activated with LPS, BzATP application for up to 30 min did not evoke measurable IL-1β release in either fraction, nor was there measurable IL-1β in either fraction at 10 or 30 min from LPS-treated cells in the absence of BzATP stimulation (n = 2); these results are similar to those found in previous studies in which longer duration stimulation has been examined (Hogquist et al., 1991; Perregaux and Gabel, 1994; Ferrari et al., 1997; Laliberte et al., 1999; Grahames et al., 1999). IL-1β appeared in the microvesicle-free supernatant with a time lag of 2–10 min behind its presence in microvesicles (Figures 6A–6C). In the absence of extracellular calcium, no IL-1β was measured at 5 or 10 min and was reduced by 80%–90% at 30 min (Figure 6A).

Western blot analysis showed that microvesicular IL-1β consisted of both the unprocessed (31 kDa) and the mature (17 kDa) form of this cytokine but any IL-1β that may have been present in the microvesicle-free supernatant (5 and 10 min time points) was below the limits of immunoblot detection (n = 3; Figure 6C). In common with many previous reports (e.g., Hogquist et al., 1991; Perregaux and Gabel, 1994; Laliberte et al., 1999; Di Virgilio et al., 1998), we detected only the unprocessed (31 kDa) but never the 17 kDa form of IL-1β in cell extracts (Figure 6C). Our findings of the prominent 17 kDa band also provide further confirmation that the rapidly released IL-1β was not the result of cell lysis and show that the bioactive form of the cytokine is present

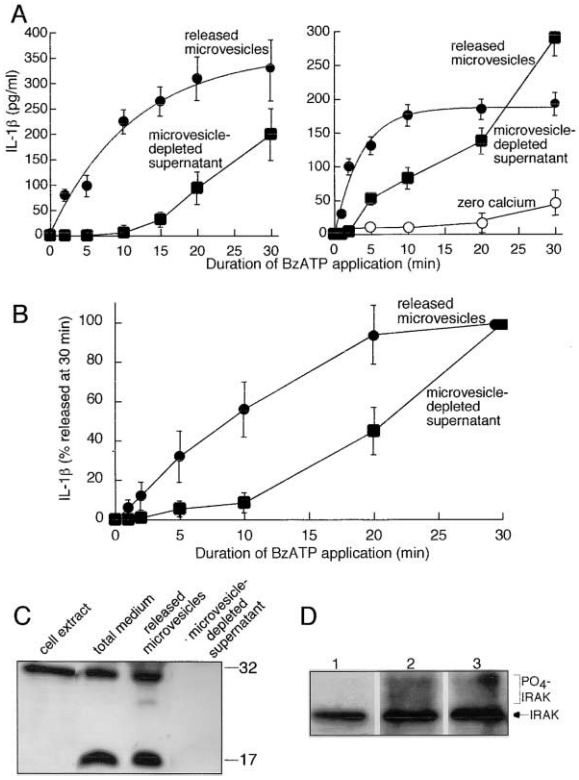

Figure 6. Bioactive IL-1β Is Present in Released Microvesicles within 1 Min of $P2X_7$ Receptor Activation and Precedes Release into Vesicle-Free Supernatant

(A) Shown are two examples of results from individual secretion experiments for IL-1β release; each point is the average ± SEM of four replicate wells, each containing 2×10^5 cells; the results are representative of a further five similar experiments in normal solution and three experiments in zero calcium.

(B) The summary of all secretion experiments plotted as percent IL-1β relative to the amount at 30 min time point; n = 7–9 for each point.

(C) IL-1β immunoblot on SDS-PAGE gel from LPS-activated THP-1 cells and medium exposed to BzATP for 10 min. Cell extract (lane 1) shows only a 31 kDa band; total medium (lane 2) obtained from 7.5×10^6 cells, concentrated through 10 kDa filter, shows both forms of IL-1β as does fraction pulled-down with annexin beads from medium obtained from a parallel set cells (lane 3). No bands (lane 4) are detected from the annexin bead-depleted supernatant obtained from the same experiment as lane 3.

(D) IRAK immunoblot of HeLa cells exposed to control medium obtained from LPS-only treated THP-1 cells (lane 1) and medium obtained from LPS-activated THP-1 cells exposed to BzATP for 10 min (lane 2) or treated with 0.5 ng/ml recombinant IL-1β (lane 3).

in the rapidly released microvesicles at least as early as 5 min.

IL-1β in Shed Microvesicles Is Bioactive

We then asked whether the fully processed IL-1β within the shed microvesicles could have any biological activity at target cells expressing IL-1 receptors. We collected medium from activated or unactivated monocytes after BzATP application, applied the medium to HeLa cells, which express IL-1 receptors, and examined the effects in two different bioassays, an interleukin-1 receptor-associated kinase (IRAK) immunoblot and a green fluorescent protein (GFP) reporter assay. Stimulation of IL-1 receptors phosphorylates IRAK within 2 min, although previous studies have only examined actions of relatively high concentrations (10–200 ng/ml) of exogenous IL-1β or IL-1α (Cao et al., 1996; Yamin and Miller, 1997; Wesche et al., 1997; Li et al., 2000). We compared IRAK immunoblots from HeLa cell extracts exposed for 5–8 min to medium obtained from LPS-treated THP-1 cells exposed to BzATP for 10 min with those directly exposed to 0.5 ng/ml recombinant IL-1β, a concentration our ELISA assays indicated was maximally present in the released microvesicles at this time point (range 150–550 pg/ml, Figure 6A). Similar amounts of phosphorylated IRAK were detected (Figure 6B). This result showed that the shed microvesicles were capable of releasing their contents onto other cells to activate IL-1 receptors and that this release of contents from the microvesicles occurred within at least 5 min. However, this method was not sufficiently sensitive to determine whether microvesicular contents released earlier were also capable of downstream bioactivity. For these studies we used HeLa cells which had been transfected with an EGFP-reporter construct containing a cytokine-responsive promoter which can detect activation of IL-1 receptors in HeLa cells by concentrations of recombinant IL-1β as low as 2–5 pg/ml (Kiss-Toth et al., 2000). We counted green cells after application of the total medium (microves icles + supernatant) or microvesicle-free supernatant (Figure 7; see Experimental Procedures). Only the medium containing microvesicles collected 2–10 min after BzATP application turned on GFP expression (Figures 7A and 7B). At the 30 min time point, there was activation of the target cells from both fractions, being consistently greater from the total medium than from the microvesicle-free medium (Figure 7B). BzATP alone did not turn on GFP expression (Figure 7B), nor did medium collected from untreated THP-1 cells (n = 4), LPS-only cells, or cells exposed to BzATP but not LPS-primed (n = 2 for each condition). IL-1 receptor antagonist (IL-1Ra, 20 μM) blocked activation by the collected medium as well as the activation by IL-1β itself (Figure 7B). These results show that the IL-1β contained in the released microvesicles at very early times can be released onto other cells in physiologically significant amounts.

Discussion

Using fluorescent video microscopy and NBD-labeled plasma membrane, we have shown that monocytes shed microvesicles of <0.5 μm diameter from their plasma membrane within 2–5 s after activation of $P2X_7$ receptors. Phosphatidylserine translocation (PS flip) preceded microvesicle shedding as measured by FITC-conjugated annexin-V binding to the plasma membrane. Microvesicle shedding was associated with a substantial decrease in cell membrane area as measured by capacitance measurements; both phenomena were dependent on extracellular calcium. PS flip after brief (≤10 min) receptor activation was reversible and was not associated with subsequent cell death for up to 24 hr. Using annexin-coated beads, we isolated microvesicles released from LPS-primed monocytes during brief $P2X_7$

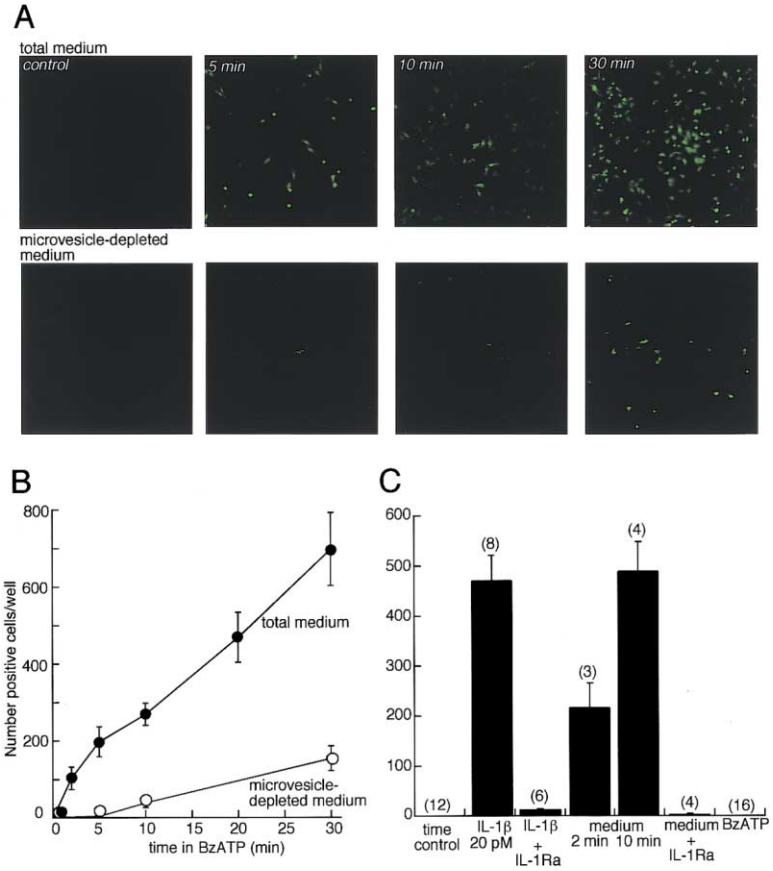

Figure 7. IL-1β in Released Microvesicles Is Bioactive

(A) Low-power fluorescent images of HeLa cells expressing IL-8 promoter-EGFP reporter construct. Total medium (upper panels) or microvesicle-free medium (lower panels) collected at time points indicated from experiments as in Figure 6 were applied to cells for 6 hr to induce activation of the cytokine-responsive promoter. Each image is 1 mm^2 of a microwell in a 96 well plate; all wells contained a confluent layer of cells by bright-field observation.

(B) Positive cells/well plotted as a function of the time after BzATP application onto THP-1 cells, at which point the medium was collected and placed onto the HeLa cells.

(C) Summary of results from IL8-EGFP reporter assay when IL-1β itself, the collected medium in the absence or presence of IL-1 receptor antagonist (20 μM), or BzATP alone (200 μM) was applied. The numbers in parentheses indicate the number of experiments. The data from medium are from a separate series of experiments from those plotted in (B).

receptor activation and measured IL-1β content; within 2 min IL-1β was present in the isolated microvesicles and only later appeared in the vesicle-free supernatant. The IL-1β in the microvesicles was bioactive, as assayed by its ability to stimulate IRAK phosphorylation in HeLa cells and transcription in HeLa cells expressing an IL8-GFP reporter gene. Because we observed the 17 kDa form of IL-1β in the microvesicular fraction on Western blots and because only the 17 kDa form of IL-1β is bioactive, we can safely assume the IL-1β released from these shed microvesicles is the mature 17 kDa molecule. We can therefore conclude that shedding of plasma membrane microvesicles containing fully processed IL-1β is the major secretory pathway for rapid release of this cytokine. It should be emphasized that our experiments do not rule out the possibility that other cytokines may be released along with IL-1β; in particular we might expect IL-1α to be secreted in this manner. However, the finding that the IL-1 receptor antagonist, which is selective for IL-1 (α and β forms) but does not block actions of other known cytokines (Dinarello, 1996), completely blocked activation of HeLa cells exposed to microvesicles released from THP-1 cells (e.g., Figure 7) means that this bioactivity we observed is due solely to activation of IL-1 receptors by released bioactive IL-1.

Two types of membrane vesicles released into the extracellular space have been demonstrated in previous studies on platelets, T cells, and/or monocytes as a result of various stimuli: exosomes derived from exocytosis of endolysosome-related multivesicular bodies, and the shedding of plasma membrane particles, which are known as microvesicles, the term we have used (Heijnen et al., 1999; Aupeix et al., 1997). Microvesicles have ubiquitously been associated with PS exposure while exosomes have not (Heijnen et al., 1999; Aupeix et al., 1997; Satta et al., 1994; Zwaal and Schroit, 1997; Dachary-Prigent et al., 1993). The decreased membrane capacitance associated with $P2X_7$ receptor activation abnegates the possibility that the PS-flipped IL-1β-containing vesicles we isolated resulted from exocytosis of exosomes, the mechanism that has previously been suggested to be responsible for a portion of IL-1β release after combined and prolonged LPS and ATP stimulation (Andrei et al., 1999). Our data provide direct (membrane capacitance) and indirect (annexin-V and NBD imaging) evidence that the source of the IL-1β-containing microvesicles released upon $P2X_7$ receptor activation is, indeed, the plasma membrane.

No previous studies of cytokine-secreting cells have measured microvesicle shedding or IL-1β release over the rapid time course we have examined, although microvesicle shedding is certainly a common occurrence in hematopoietic and other immune cells. Earlier workers have isolated biochemically (Satta et al., 1994; Zwaal and Schroit, 1997; Dachary-Pringet et al., 1993; Martinez-Lorenzo et al., 1999) or observed by electron microscopy (Denzer et al., 2000) membrane microvesicles of 0.1–0.4 μm diameter in the supernatant of monocytes after several hours of LPS stimulation (Satta et al., 1994) or T cells after as short as 15 min stimulation with anti-

CD3 or PHA (Martinez-Lorenzo et al., 1999). These microvesicles could be labeled with annexin-V, were found to possess a number of MHC class II proteins, integrin molecules such as P selectin and Fas ligand, and were greatly increased after treatment with the calcium ionophore, A23187, although they were not assayed for presence of cytokines (Satta et al., 1994; Zwaal and Schroit, 1997; Dachary-Pringent et al., 1993; Denzer et al., 2000; Gu et al., 1998; Martinez-Lorenzo et al., 1999). In these previous studies, microvesicles were isolated by ultracentrifugation, which would have expelled any vesicular contents such as IL-1β, since we found all but the most gentle centrifugation of the microvesicles we isolated resulted in loss of IL-1β from the vesicular fraction. In common with results from several previous studies (Hogquist et al., 1991; Perregaux and Gabel, 1994; Ferrari et al., 1997; Sanz and Di Vrigilio 2000, Laliberte et al., 1999; Grahames et al., 1999), we observed that IL-1β release was minimal or undetectable after LPS stimulation alone and after BzATP stimulation alone. We found IL-1β in microvesicles within 1 min of receptor activation in LPS-primed monocytes and the fully processed 17 kDa molecule was detected within 5 min of $P2X_7$ receptor activation. A23187 does not cause release of bioactive 17 kDa IL-1β from LPS-primed macrophage (Perregaux et al., 1992; Bahl and Foreman, 1994). Nevertheless, the process of rapid microvesicle shedding required only activation of $P2X_7$ receptors and extracellular calcium whether or not the vesicles contained IL-1β (i.e., it occurred in HEK cells as well as in non-LPS-treated THP-1 cells). Thus, it seems likely that the antigen-presenting microvesicles isolated in previous studies are the same as the microvesicles isolated in our study but only when $P2X_7$ receptors are engaged do these microvesicles contain processed IL-1β. It remains to be determined whether, indeed, these are the same population of microvesicles, and/or what other molecules are expressed on the surface of, and contained within, the rapidly shed microvesicles we have now identified and shown to contain bioactive IL-1β. However, the fact that these microvesicles are associated with PS exposure provides the means for their specific targeting to PS receptor-expressing cells in a manner similar to that well known for platelet aggregation and procoagulant activity (Zwaal and Schroit, 1997; Allen et al., 1988). Dendritic cells in particular express the PS receptor (Fadok et al., 1992, 2000), and IL-1β stimulation of dendritic cells is the first step toward initiation of the acute inflammatory response (Hart, 1997); thus, these rapidly released PS-flipped microvesicles may be targeted specifically for dendritic cells.

Release of mature (17 kDa) bioactive IL-1β evoked by $P2X_7$ receptor activation in LPS-stimulated monocytes and microglia has been suggested to result from an active process involved with apoptosis and subsequent death of the releasing cell (see Di Virgilio et al., 1998). Although it is likely that much of the IL-1β released after prolonged stimulation of $P2X_7$ receptors may be from microvesicles shed from late apoptotic cells, we found no evidence for apoptosis, necrosis, cell lysis, or membrane breakdown in cells exposed to BzATP for up to 10 min. Indeed, PS flip was reversible within 3 hr after these brief BzATP applications. PS flip has been a commonly accepted hallmark of early events of apoptotic cells, but our results and another recent study (Hammill et al., 1999) show that this is not a reliable marker of cells destined for cell death. Instead, it may, in some circumstances, indicate a cell engaged in membrane shedding of secretory proteins.

How the released microvesicles ultimately release IL-1β onto interleukin receptors of other cells remains for future studies. It may be difficult to approach this by studies on monocytes in tissue culture conditions, where release occurs into a virtually infinite solution, or in vivo, especially at sites of inflammation, where the dissemination of microvesicles may be highly restricted. In the present study, IL-1β in the microvesicular fraction reached a plateau after 10–20 min, while that in the vesicle-free supernatant continued to increase over the 30 min time period examined. This could indicate distinct release processes, one from a "readily releasable" pool of microvesicles and another from endolysosomes (Andrei et al., 1999) or an as-yet-unidentified mechanism, or it could simply be due to microvesicle disruption during time in the extracellular solution. The clear conclusion that we can make from our IL-1β ELISA measurements and IL8-GFP reporter bioassay results is that bioactive IL-1β is present in released microvesicles at least as early as 2 min after $P2X_7$ receptor activation (e.g., Figures 6 and 7). Such rapid release of this key initiator of the acute inflammatory response has not been appreciated previously, although recent in vivo experiments have commented on the unexpectedly rapid release of IL-1β in neurotrauma (Fassbender et al., 2000). Can we reconcile our new findings of rapid IL-1β release without subsequent cell death with previous findings of cell death, release of endolysosomes, and IL-1β release when receptor activation is prolonged? One may speculate that these events represent two ends of a continuum based on similar underlying mechanism(s). These events may involve time-dependent recruitment of distinct associated proteins (Schilling et al., 1999), pore dilatation (Virginio et al. 1999a, 1999b), or other second messenger pathways (Di Virgilio et al., 1998).

In summary, our data suggest the following sequence of events. Following priming of the cytokine-caspase cascade by the initial inflammatory stimulus, activation of $P2X_7$ receptors by extracellular ATP causes PS flip and loss of plasma membrane asymmetry within a few seconds. Some seconds later, PS-exposed microvesicles containing bioactive IL-1β pinch off from the cell, and this IL-1β is able to activate IL-1 receptors on other cells. The rapidity of the IL-1β release is appropriate to its role as the initial player in the inflammatory cytokine cascade, but it might also indicate signaling roles in processes other than inflammation. In a broader sense, the results suggest that the microvesicle formations and release might be a more general pathway for the secretion of cytoplasmic secretory proteins that lack leader sequences.

Experimental Procedures

Cells and Reagents

HEK293 cells stably expressing the rat $P2X_7$ receptor have been described (Virginio et al., 1999a). Human THP-1 monocytes were differentiated with 0.5 μM phorbol-12-myristate 13-acetate (PMA)

for 3 hr and then plated onto coverslips for single-cell experiments and into 24 well plates or 35 mm petris for IL-1β release experiments (Grahames et al., 1999). Transient transfection with GFP was performed by using lipofectin as previously described (Virginio et al. 1999a); transfection yielded GFP-positive cells in >80% of HEK cells and <2% of THP-1 monocytes. Biotin and FITC-conjugated annexin-V, 2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD) and 2′,7′-bis-(carboxyethyl)-5-(6′)-carboxyfluorescein (BCECF-AM) were obtained from Molecular Probes. Streptavidin-agarose beads, 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP) and lipopolysaccharide (LPS) were from Sigma; recombinant human IL-1β was from Endogen. A concentration of BzATP producing approximately 90% of maximum response (EC_{90}) was used in all experiments described herein; this was 30 or 100 μM for HEK293 cells heterologously expressing $P2X_7$ receptors and 200 or 300 μM for THP-1 monocytes (Mackenzie et al., 1999; Surprenant et al., 1996; Hibell et al., 2000; Grahames et al., 1999).

Microscopy and Electrophysiology

Standard whole-cell recordings and digital video microscopy using a Zeiss Axiovert 100 with Fluar X20, X40, or X100 objective, HEKA EPC9 with Pulse software, and the Photonics monochromator (TILLion VISION) system were as described previously (Virginio et al., 1999a, 1999b). Extracellular solution contained (in mM): NaCl 147 or NMDG 147, KCl 2, $MgCl_2$ 1, $CaCl_2$ (2 or 0 with EGTA), Hepes 10, glucose 12; intracellular solution (mM) was: NaCl 147, Hepes 10, EGTA 10 (pH 7.3, osmolarity 300 mOsm). Hypertonic solution contained 270 mM sucrose. We were unable to track membrane capacitance during $P2X_7$ receptor activation using currently available methods (Lollike and Lindau, 1999) because of the massive increase in conductance (from resting value of approximately 50 pS to >100 nS in HEK cells and from about 100 pS to 10–60 nS in THP-1 cells); therefore, we recorded membrane capacitance values 15 min after establishment of whole-cell recording prior to any stimulation and 1 min after cessation of BzATP application. NBD labeling of plasma membrane was achieved by incubation with 5–10 μM for 2–5 min at room temperature; fluorescent imaging was carried out in the subsequent 5–10 min. NIH Image 1.62 software was used to make Quicktime movies from digital images captured at 0.2–2 Hz; all movies show 1–4 frames prior to agonist application and the subsequent 2 min. Measurements of NBD-labeled microvesicle shedding and annexin binding were made in a nonperfused bath for better resolution of released vesicles, but because of this and because images were recorded in a single focal plane, it is not possible to obtain an accurate count of the total number released from one cell nor to make interpretations concerning subsequent movements of these particles. We have counted as microvesicles only those fluorescent particles that unequivocally moved from the membrane edge to >5 μm away from cell or out of the field of view and have ignored any fluorescent movements that appears to remain attached to any part of the cell. For scanning electron microscopy, cells were processed as described previously (Hallam and Rink, 1985) and viewed with a Philips XL 30 FEG scanning electron microscope.

IL-1β and LDH Release

PMA-differentiated THP-1 cells (2×10^5 cells/well in 24 well plate) were treated for 2 or 6 hr with the endotoxin LPS (10 μg/ml). Cells were washed twice with extracellular solution, and BzATP (200 or 300 μM) was applied for time periods between 1–30 min. Supernatant was collected and gently mixed for 10–30 min with biotin-annexin-coated streptavidin-agarose beads. Preliminary experiments using the 30 min time point were conducted to determine saturating concentrations of annexin to ensure complete pull-down of PS-exposed microvesicles; this was 20 μl annexin/20 μl beads/150 μl sample. We also compared IL-1β from supernatants exposed to beads only with those from annexin-coated beads; ELISA assays showed IL-1β from beads-only fraction was 0%–10% of IL-1β obtained from annexin-coated beads fraction ($n = 2$, 10 min time point). These results confirmed that annexin was required for effective pull-down of IL-1β-containing microvesicles. Beads were separated from supernatant by gentle spin (1200 rpm/3 min) in initial experiments or by allowing to settle for 30 min in later experiments; the latter protocol yielded significantly greater values for IL-1β content in the vesicular fraction than the bead-free supernatant, suggesting that even this gentle spin was sufficient to cause some release of vesicular contents. Fractions containing the beads were treated with 2% Triton X to release vesicle contents prior to ELISA. Because annexin V binds to PS only in the presence of calcium (Andree et al., 1990), release experiments in zero calcium solution were analyzed by treating the total medium (microvesicles + supernatant) with 2% Triton X and then assaying for IL-1β levels. Vesicular and vesicle-free supernatant fractions were assayed for IL-1β using the ENDOGEN human IL-1β ELISA kit or with the R&D Systems human IL-1β Quanti-Glo ELISA kit. Supernatant or triton-lysed cells or microvesicles were assayed for LDH content by using the CytoTox 96 assay kit (Promega). In some experiments, medium was collected from LPS-treated THP-1 cells after stimulation with BzATP for 15 min and incubated with supramaximal concentrations of trypsin (1 μg/10^5 cells) at 37°C for 15–40 min, after which microvesicle fraction was collected by the annexin bead pulldown and assayed for IL-1β. Exogenous recombinant IL-1β (1.5–3 ng) was completely digested by a lower amount of trypsin (100 ng) within 15 min, as assayed by Western blotting; this served as the control for the protease digest experiments.

Immunoblots

Supernatants from LPS-treated THP-1 monocytes (7.5×10^6 cells) were withdrawn following activation for 5 or 10 min with 300 μM BzATP and were either passed through annexin-coated beads as used for ELISAs or immediately concentrated using 10 kDa nominal molecular weight cutoff filters (Flowgen) and then lysed in SDS-PAGE loading buffer containing protease inhibitors (Cocktail III, Calbiochem). THP-1 cell extracts were prepared by lysis in PBS + 1% NP40 with protease inhibitors, followed by centrifugation to remove particulate material. Protein bound to the annexin-coated beads was eluted by the addition of SDS-PAGE loading buffer and heating to 95°C. Cell extracts and concentrated supernatants were analyzed on a 12% polyacrylamide gel and transferred onto PVDF membrane. IL-1β was detected by using a polyclonal human anti-IL-1β Ab (Santa Cruz Biotech, sc-7884). IRAK assay was the same as described in detail previously (Yamin and Miller, 1997; Li et al., 2000). Briefly, supernatants from 6×10^6 THP-1 cells were withdrawn following activation for 10 min with 300 μM BzATP and immediately added to 5×10^6 HeLa cells for 5–8 min, in the presence of inhibitors of proteasome activity (Peptides International, Louisville, KY). Extracts were harvested immediately by scraping cells in lysis buffer and solubilized for 2 hr at 4°C. IRAK was immunoprecipitated using 5 μl of polyclonal anti-IRAK Ab (Upstate Biotech, Lake Placid, NY) by overnight incubation, followed by addition of protein G sepharose (Amersham Phramacia Biotech) for 2 hr. Samples were spun and washed four times in lysis buffer. Bound protein was eluted in 40 μl SDS-PAGE loading buffer by heating to 95°C. Extracts were analyzed on a 10% polyacrylamide gel and transferred onto PVDF membrane, and IRAK was detected with IRAK Ab.

Green Fluorescent Protein (GFP) Reporter Assay

Methods have been described in detail previously (Kiss-Toth et al., 2000). Briefly, the human IL8 promoter was subcloned into the pd2EGFP1 vector (Clontech) to yield pIL8/d2EGFP1. This cDNA (0.5 μg/well) was used to transfect 90% confluent layers of HeLa cells, plated into 96 well plates with SuperFect (Qiagen); control wells were similarly transfected with pEGFP1 vector. Twenty-four hours after transfection, each well was viewed with a 10× fluorescence objective to count background green cells; no well transfected with pIL8/d2EGFP1 had >4 green cells ($n = 225$ wells from 5 separate transfections), while pEGFP1-transfected wells had 2650 ± 256 green cells ($n = 20$ wells from 5 transfections). Culture medium was then removed and test medium (100 μl) was added; cells were incubated at 37°C for a further 6 hr, at which time green cells were again counted. Examination of wells under bright field at this time revealed that cells in all wells remained confluent; therefore we assumed total cells/well were the same and did not change during incubation with test medium. We were not able to carry out this assay using the isolated microvesicles alone because the annexin-coated beads, at the concentration used for our pull-down assay,

caused disadherence of the HeLa cells and obscured visibility; therefore, we compared effects of total medium with vesicle-free medium. Images (512 × 512 pixels) were obtained using the 10× objective on the Nikon PCM 2000 inverted confocal microscope; no background subtraction or other processing have been applied to the images.

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